On the move: Redox –dependent protein relocation in plants

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Abbreviations: ER: endoplasmic reticulum; CAT: catalase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; NPR1: NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1; PR: pathogenesis-related; PRX: peroxiredoxins; PTM: post-translational modifications; ROS: reactive oxygen species; RNS: reactive nitrogen species, SA: salicylic acid; SAR: systemic acquired resistance; SOD: Superoxide Dismutase; TRX: thioredoxin ; UPS: Ubiquitin-proteasome system; WHY1: WHIRLY1

Figures: 4 plus 1in Box 1 (Figure 5)

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ABSTRACT

Compartmentation of proteins and processes is a defining feature of eukaryotic cells. The growth and development of organisms is critically dependent on the accurate sorting of proteins within cells. The mechanisms by which cytosol-synthesized proteins are delivered to the membranes and membrane compartments have been extensively characterised. However, the protein complement of any given compartment is not precisely fixed and some proteins can move between compartments in response to metabolic or environmental triggers. The mechanisms and processes that mediate such relocation events are largely uncharacterized. Many proteins can in addition perform multiple functions, catalyzing alternative reactions or performing structural, non-enzymatic functions. These alternative functions can be equally important functions in each cellular compartment. Such proteins are generally not dual targeted proteins in the classic sense of having targeting sequences that direct *de novo* synthesised proteins to specific cellular locations. We propose that redox post-translational modifications (PTMs) can control the compartmentation of many such proteins, including antioxidant and/or redox associated enzymes.

INTRODUCTION

Metabolic regulation is shaped by compartmentalization in all cells. Compartmentalization is required to achieve the stable metabolic states that underpin different cell fates (Harrington *et al.*, 2013). Within this context, many proteins perform multiple apparently unrelated functions, often in different locations. These are often called moonlighting proteins, the classic definition of which is proteins with two or more different functions, excluding those arising from gene fusion, homologous non identical proteins, splice variants, proteins with different post translational modifications (PTMs) and those with a single function but active in different locations or on different substrates (Jeffery, 1999). However, the number and diversity of proteins that either can have different functions in the same intracellular compartment or that can move from one compartment to another to fulfil different functions has increased enormously in recent years, aided by development and application of bioinformatic (Chapple *et al.*, 2015) proteomic and cell imaging techniques (Chong *et al.*, 2015; Thul *et al.*, 2017). Such studies have revealed that up to 50% of cellular proteins exist in multiple subcellular localisations, which can change in response to appropriate triggers (Chong *et al.*, 2015), such as disease states like cancers in animals (Min *et al.*, 2016) and stress responses in plants (Sun et al. 2018) . Several metabolic enzymes are known to move into the nucleus affecting epigenetic modifications (Boukouris *et al.*, 2016) and histone expression (He *et al.*, 2013) providing a link between metabolism and gene expression. Plant organellar proteins such as MUTS HOMOLOG1 (MSH) 1, which is a DNA-binding nucleoid protein, function in the creation of epigenetic stress memories in plants that are associated with organellar redox changes (Virdi *et al.*, 2015; Xu *et al.*, 2012). Whilst many studies have been conducted on yeast and mammalian cells, there is also incontrovertible evidence for proteins with multiple, largely unrelated functions in plants (**Table I**). For example, recent studies have identified a number of metabolic enzymes as members of the RNA binding protein repertoire (Marondedze *et al.*, 2016).

It is important to note that not all proteins that move compartments exhibit moonlighting functions, and not all proteins with such properties move compartments. For example, L-galactono-1, 4-lactone dehydrogenase has dual functions in plant mitochondria. As an enzyme it is responsible for the synthesis of ascorbic acid, and as a chaperone it is essential for the assembly of respiratory complex I (Schimmeyer *et al.*, 2016). Similarly plastid NAD dependent malate dehydrogenase has a non-enzymatic function stabilising the FtsH12 component of the inner envelope AAA ATPase (Schreier *et al.*, 2018). Proteins such as peroxiredoxins (PRX)

that readily undergo redox PTMS in their roles as ROS scavengers and oxidases have evolved to support multiple functions (acting as peroxidases, signalling proteins and chaperones) under optimal and stress conditions (Chen *et al.*, 2018). Like other redox proteins, whose functions are supported by thiol-based biochemistry, PRX can interact with multiple cellular partners in animals and plants, from thioredoxins (TRX) to transcription factors (Liebthal *et al.*, 2018).

Regulated protein relocation between the different compartments of the cell provides a robust and flexible mechanism for metabolic, genetic and epigenetic regulation in response to metabolic stimuli and environmental cues. Such responses often entail shifts in cellular redox homeostasis that lead to both oxidative and reductive events that shift protein functions and compartmentation. One important paradigm for such redox-related changes in plants is Non-expresser of pathogenesis related proteins (NPR)1, which is a master regulator of salicylic acid (SA)-mediated systemic acquired resistance (SAR) leading to broad-spectrum disease resistance in plants (Mou *et al.*, 2003). NPR1 is similar to the immune co-factor I k B and the transcription factor NF-k B in mammals, suggesting that there is conservation of immune responses (Sun *et al.*, 2018). In the cytosol, NPR1 exists in a large disulfide-bonded oligomeric complex. Stress-induced SA accumulation leads to reduction of the intermolecular disulfide bonds within the complex by TRX (Tada *et al.*, 2008) and release of the NPR1 monomers. These are then phosphorylated in the cytosol and imported into the nucleus (Mou *et al.*, 2003). Further phosphorylation of NPR1 in the nucleus promotes interactions with transcription factors such as WRKY and TGA in a redox-dependant manner leading to the expression of PR genes. Other proteins that move from the cytosol to the nucleus upon perturbation of redox homeostasis are known in yeast. The AP-1-like transcription factor (YAP1), which a member of the basic leucine zipper protein family (bZIP), translocates when oxidized from the cytosol to the nucleus, where it activates genes encoding oxidative stress tolerance proteins (Kuge *et al.*, 1997), while the enzyme Superoxide Dismutase, SOD1, move to the nucleus to moonlight as a transcription factor (Tsang *et al.*, 2014). Examples from mammalian cells include NRF2, CLK-1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reviewed in (Min *et al.*, 2016; Monaghan and Whitmarsh, 2015).

A second paradigm is organelle-to-nucleus retrograde signalling pathways that allow cells to adapt to changes in metabolic state, often in a redox-dependent manner (Boukouris *et al.*, 2016; Monaghan and Whitmarsh, 2015). In this Expert View, we discuss plant proteins that are

known or suspected to move location in the cell to perform alternative activities. We also present evidence in support of the hypothesis that redox cues and resulting PTMs not only alter the interactome of redox-sensitive proteins but also central to mechanisms that facilitate the movement of proteins between compartments.

MECHANISMS OF PROTEIN MOVEMENT IN PLANTS

ROS triggered post translational modifications (PTMS)

Reduction-oxidation (redox) processes not only drive cellular energy metabolism but they are crucial to cell signalling and communication (Foyer and Allen, 2003). A vast wealth of molecular genetic evidence supports the concept that reactive oxygen species (ROS) produced by photosynthesis, respiration and other metabolic processes, and by specific enzymes such as NADPH oxidases are essential signalling molecules that control plant growth and stress responses (Foyer, 2018) (Mhamdi and Van Breusegem, 2018; Schmidt and Schippers, 2015). This is achieved by redox-mediated PTMs of the cysteines residues on key proteins involved in multiple pathways such as primary and secondary metabolism, cell cycle phytohormone metabolism and signalling, gene expression, translation and protein production and transport. Chloroplasts, mitochondria and peroxisomes are thus not only the essential sites of metabolic energy production and utilization, but also important sources of ROS and other redox regulators that influence nearly every aspect of cell biology (Noctor and Foyer, 2016). While cells regulate redox processes in a compartment-specific manner, redox PTMs may also be used to regulate the movement of proteins between compartments, as described for NPR1 above (Tada *et al.*, 2008). Assessing the protein-protein interactions that are involved in the functions of NPR1 and other redox regulated proteins is not only technically challenging but also entails considerations of the inter-dependent facets of redox state and oligomeric structure. Moreover, ROS and redox cues modify microtubule orientation and behaviour within plant cells (Dang *et al.*, 2018), as well as the operation of protein import machineries (reviewed in (Bolter *et al.*, 2015; Ling and Jarvis, 2015).

Redox PTMs on protein cysteines are formed enzymatically or non-enzymatically via promiscuous reactive species, including ROS, reactive nitrogen species (RNS), and other radicals or electrophilic lipids. There is growing appreciation that redox PTMs are site-specific,

governed by the microenvironment of the cysteine residues, and that they are subject to temporal and spatial control. Studies using small molecule and protein-based fluorescent sensors have shown that eukaryotic cells tightly control the location of reactive species, proteins and redox state across compartments (Kaludercic *et al.*, 2014). However, rather than being fixed, this balance is flexible and responsive to metabolic and environmental controls. For example, it is shifted during the ageing processes in the model organism *C. elegans* (Kirstein *et al.*, 2015). As in other organisms, redox PTMs control the activities and binding partners and probably also the compartmentation of many plant proteins, including antioxidant and/or redox associated enzymes (**Box I**), as discussed in detail below.

Protein Import and Export

The molecular mechanisms of protein import into mitochondria, chloroplasts and peroxisomes have now been established and the importance of the accuracy of these processes underscored by the realization that defects result in human disease. Recent work has revealed that protein import can be regulated at several levels; from modification of individual precursor proteins to prevent or alter their targeting, to regulated interaction with binding partners, and modification of the import apparatus by phosphorylation or ubiquitination to alter its activity (Bolter *et al.*, 2015; Harbauer *et al.*, 2014; Ling *et al.*, 2012) (**Figure 1**). Such processes allow the location of proteins to change in response to changes in cellular state. For example, in *C. elegans* the transcription factor ATF1 is imported into mitochondria and degraded by a Lon protease but, when import is decreased, ATF1 relocates to the nucleus and induces an unfolded protein response (Nargund *et al.*, 2012). In mammals, import of the protein catalase into the peroxisome is redox regulated and under stress conditions the peroxisome import receptor PEX5, retains catalase in the cytosol (Walton *et al.*, 2017). Intriguingly, an old observation that NADPH but not NADH inhibits protein import hints at the importance of redox balance for protein import into plant peroxisomes as well (Pool *et al.*, 1998). Retrograde signalling from organelles to the nucleus to integrate cellular activities is well established, and modulation of chloroplast import activity is important in response to biotic and abiotic stress (de Torres Zabala *et al.*, 2015; Ling and Jarvis, 2015).

Proteins are exported from mitochondria, chloroplasts and peroxisomes and the endoplasmic reticulum (ER) (**Figure 1**). The best characterised mechanisms of protein export are involved

in protein degradation and in organelle quality control via the cytosolic Ubiquitin-proteasome system (UPSl (Bragoszewski *et al.*, 2017; Kao *et al.*, 2018; Ling and Jarvis, 2016). In peroxisomes, for example, an ER associated degradation (ERAD)-like system exports the import receptor PEX5 from the peroxisome membrane to perform further rounds of import. PEX5 cycling between peroxisome and cytosol is regulated by ubiquitination of a conserved Cys, and in mammalian cells reduced glutathione can de-ubiquitinate the receptor (Grou *et al.*, 2009). Although not shown experimentally, this Cys is conserved in plant PEX5 proteins suggestive of a similar mechanism operating. Mutants in this PEX 5 re-export system in Arabidopsis also fail to degrade some peroxisome matrix proteins suggesting they are exported for degradation (Burkhart *et al.*, 2013). The release of transcription factors from cellular membranes by regulated proteolysis is also a well-known response to stress in both animals and plants (Seo *et al.*, 2008; Sun *et al.*, 2011). Potentially, protein export from organelles and retargeting (rather than degradation) could provide a means of signalling and genetic regulation. To date this has only been proposed/described for a handful of proteins and the mechanism(s) by which this occurs and is regulated are still obscure (Foyer *et al.*, 2014). The next section presents proteins which are candidates for regulated relocation.

Candidates as a paradigm for redox regulated movement in plants

GAPDH is a quintessential example of a moonlighting protein (Sirover, 2012, 2014). Several GAPDH isoforms exist in different subcellular localizations in plants (Holtgrefe *et al.*, 2008). In animals, it has multiple functions in addition to its classic role in glycolysis, such as DNA stability and control of gene expression, autophagy and apoptosis. Both the activity and localization of the plant cytosolic GAPDH isoform (GapC) are controlled by cellular redox state (Bedhomme *et al.*, 2012). Redox PTMs on the cytosolic GAPDH protein in animals block enzyme activity and promote novel cell signalling and transcription functions in the nucleus (Yang and Zhai, 2017) (Zaffagnini *et al.*, 2013). The functions of GAPDH in the nuclei of plant cells are not clear, but nuclear GAPDH may have a role as a coactivator for gene expression (Hildebrandt *et al.*, 2015). Since GapC is also localized in the nucleus, it is suggested that redox modification facilitates transfer to the nucleus in plants as it does in animals (Ortiz-Ortiz *et al.*, 2010). However, the mechanism of nuclear translocation of GapC is unknown although it is thought to involve *S*-sulfhydration, a process that reversibly regulates the function of this protein, in a manner similar to that described in mammalian systems (Aroca

et al., 2015) . However, GapC undergoes *S*-nitrosylation, *S*-glutathionylation, *S*-sulfhydration, *S*-sulfenylation as well as other modifications that all occur on the same cysteine residue (Aroca *et al.*, 2017; Bedhomme *et al.*, 2012; Lindermayr *et al.*, 2005; Waszczak *et al.*, 2014). Thus, how each type of PTM modifies GapC to shift location and/or alternative instigate non-metabolic functions remains to be determined.

Catalase (CAT) is a peroxisomal enzyme whose import in mammals is redox-regulated (Walton *et al.*, 2017) and in yeast is dependent on carbon source (Horiguchi *et al.*, 2001). In plants it is classically known as a peroxisomal enzyme but recent evidence suggests that the compartmentation of this central antioxidant enzyme may be more dynamic than the literature acknowledges. The role of CAT as a central 'redox guardian' is well established (Mhamdi *et al.*, 2012). Plant catalases have been shown to interact with a variety of cytosolic proteins including calmodulin (Yang and Poovaiah, 2002), calcium-dependent protein kinase 8 (CDPK8) (Zou *et al.*, 2015), salt overly sensitive 2 (SOS2) (Verslues *et al.*, 2007), lesion stimulating disease1 (LSD1) (Li *et al.*, 2013), receptor like cytoplasmic kinase STRK1 (Zhou *et al.*, 2018) and no catalase activity 1 (NCA1) (Hackenberg *et al.*, 2013; Li *et al.*, 2015) (**Figure 2**). All are integral stress signalling proteins. The *nca1* mutants, which lack a functional CAT, are hypersensitive to abiotic stresses. Similarly, the *cat2* mutant of Arabidopsis, which lacks the predominant leaf isoform that is essential for the metabolism of H_2O_2 produced by photorespiration, activates a wide range of salicylic acid (SA) and jasmonic acid (JA)-dependent responses and displays day-length dependent localised programed cell death (PCD) and resistance to pathogens (Queval *et al.*, 2010). CAT can also be a target for pathogen encoded-effector proteins (Mathioudakis *et al.*, 2013; Murota *et al.*, 2017). The fungal effectors PsCRN115 and PsCRN63 both traffic CAT to the nucleus but have opposite biochemical and physiological effects. PsCRN115 stabilises catalase, decreases H_2O_2 and reduces PCD, whereas PsCRN63 destabilises catalase increases H_2O_2 and increases PCD (Zhang *et al.*, 2015). How can the interaction of peroxisomal catalase with such a wide variety of cytosolic proteins be explained? Some interactions may be occurring during biosynthesis in the cyotsol before import into peroixisomes. However the evidence that catalase interacts with different stress signalling and PCD proteins potentially provides a mechanism for protein retention and/or relocation. We speculate that the location of cytosolically-synthesised CAT is determined by competition among different potential-binding partners as a consequence of reduced import into peroxisomes and/or increased retention of CAT in the cytosol. While sensitivity of peroxisomal protein import to redox status is likely to impact import of all peroxisome proteins,

CAT which has a non-canonical targeting signal (Mhamdi *et al.*, 2012) (Rymer *et al.*, 2018) may be more sensitive and indeed PEX5, the major peroxisome import receptor, has been proposed to specifically retain mammalian catalase in the cytosol under conditions of oxidative stress (Walton *et al.*, 2017). This property, combined with the potential to interact with an array of cytosolic proteins as shown in **Figure 2** could allow swift control of catalase localisation between compartments in such a way as to influence various redox signalling pathways.

WHIRLY1 (WHY1) is a member of a small family of ssDNA binding proteins that are specific to the plant kingdom (Desveaux *et al.*, 2005; Desveaux *et al.*, 2004). WHY1 protein is encoded in the nuclei and targeted to chloroplasts and the nuclei, the nuclear and processed chloroplast forms having the same molecular mass (Grabowski *et al.*, 2008). Studies using epitope tagged, transplastomically-expressed WHY1 provided evidence that WHY1 can move directly from the chloroplasts in the nuclei (Isemer *et al.*, 2012): (Foyer *et al.*, 2014). However, the factors that trigger and regulate this apparently direct movement of this protein from the chloroplasts to the nuclei are unknown. Posttranslational modification of WHY1 in the cytosol can also regulate the partitioning between the chloroplasts and nuclei. This change in partitioning is regulated by phosphorylation of WHY1 in the cytosol by a serine/threonine SNF1-related protein kinase called calcineurin B-Like-Interacting Protein Kinase14 (CIPK14). Phosphorylation of WHY1 results in transport to the nucleus (Ren *et al.*, 2017). The phosphorylation of WHY1 in the cytosol regulates the intracellular localization with respect to leaf development. WHY1 being predominantly in the chloroplasts of young leaves, while in senescing leaves the protein is localized mainly in the nucleus (Ren *et al.*, 2017). It is perhaps not surprising therefore that WHY1 is a multifunctional protein, with DNA binding properties that are relevant in both cellular compartments. WHY1 is important in the regulation of chloroplast development, plastome copy number and plastome gene expression, chloroplast ribosome formation and chloroplast to nucleus signaling (Comadira et al., 2015; (Comadira *et al.*, 2015; Prikryl *et al.*, 2008). WHY1 promotes ribosomal RNA splicing that is catalysed by other factors within plastids (Prikryl *et al.*, 2008). Nuclear WHY1 is involved in the expression of senescence and defence genes as well as in the maintenance of telomeres (Yoo *et al.*, 2007).

ROXY proteins. The glutaredoxins ROXY1 and ROXY2 are found in both the nuclei and cytosol (Delorme-Hinoux *et al.*, 2016). ROXY1 interacts with the TGA transcription factor called TOPLESS in the nuclei, in a redox-dependent manner, and with four other TGA transcription factors. While there is as yet no direct evidence of the redox-regulated movement

of ROXY1 between the nucleus and cytosol**,** there is no other explanation for the dual compartmentation of this protein.

Heat shock factor (HSF) A8: Like other HSFs, HSFA8: is retained in the cytosol in an inactive form by interaction with heat shock proteins (HSP)s, which mask the nuclear location signal and the oligomerisation domain. In response to oxidative and other stresses HSFs oligomerize and are translocated into the nucleus, where they modulate the expression of target genes (Scharf *et al.*, 2012). Redox-mediated nucleocytoplasmic shuttling has been characterised HSFA8 in *Arabidopsis thaliana* (Giesguth et al., 2015) Cys24, which is located in the DNA binding domain of AtHSFA8 and Cys269, which is located in the C-terminal part of the protein act as redox sensors Disulphide bond formation between Cys24 and Cys269 is thought to induce release from multi-heteromeric complexes and translocation into the nucleus (Giesguth *et al.*, 2015)

Membrane bound transcription factors. Membrane located proteins can be cleaved from their membrane anchor in response to an appropriate signal to release a soluble domain that can be relocated (**Figure 1**). Often these proteins function as transcription factors once liberated from the membrane. *ANAC013* and *ANAC017* encode Arabidopsis transcription factors belonging to the *NON APICAL MERISTEM/ARABIDOPSIS TRANCRIPTION ACTIVATION FACTOR/CUP SHAPED COTYLEDON* (*NAC*) family. These transcription factors mediate ROS-related retrograde signalling originating from mitochondrial complex III. Both proteins are anchored to the endoplasmic reticulum membrane. They were identified via one hybrid assays as binding to a conserved cis acting regulatory sequence termed the mitochondrial dysfunction motif (MDM) which mediates mitochondrial retrograde regulation (MRR) during oxidative stress (De Clercq *et al.*, 2013). GFP-ANAC013 was partially processed and nuclear localised, but while difficult to detect there was a suggestion that the full length protein is ER targeted (De Clercq *et al.*, 2013). *ANAC017* was also identified in a screen for loss of response to mitochondrial dysfunction (Ng *et al.*, 2013). It is targeted to the ER and dual tagging experiments showed it is cleaved upon antimycin A treatment which inhibits the mitochondrial electron transport chain at complex III. The N terminal part of ANAC017 locates to the nucleus whilst the C terminal part remained ER associated. *ANAC017* function was essential for hydrogen peroxide mediated stress signalling (Ng *et al.*, 2013). Upon perception of redox signals, *ANAC013* and *ANAC017* are released from the ER and translocated to the nucleus, where they activate MDS genes such as alternative oxidases (*AOXs*), *SOT12*, and *ANAC013*.

The latter provides positive feedback regulation of the signalling pathway with enhancement of the signal. The ROS-dependent signalling pathways from chloroplasts and mitochondria merge at RADICAL-INDUCED CELL DEATH1 (RCD1), a nuclear protein that is suggested to suppress the activities of the ANAC013 and ANAC017 transcription factors (Shapiguzov *et al.*, 2019). Another member of the family, ANAC089, is an ER and TGN localised membrane protein. Upon treatment with reducing agents the N terminal domain of ANAC089 localises to nuclei where it partially supresses chloroplast stromal ascorbate peroxidase gene expression. (Klein et al., 2012).

The chloroplast bound plant homeodomain transcription factor PTM was proposed to play a crucial role in chloroplast signalling to the nucleus. Full length PTM is located to the chloroplast outer envelope where as a truncated form lacking the TM domain was nuclear. Treatments such as high light and Norfluazon were reported to result in cleavage of PTM and localisation of the N terminal domain to the nucleus. Processed PTM was shown to activate ABI4 transcription (Sun *et al.*, 2011). Mutants defective in this gene show aberrant responses to treatments such as Norfluazon, high light dibromothymoquonine and Rose Bengal that affect different ROS and the level of reduction of the plastoquinione pool (Sun et al., 2011), although these results were not observed in a subsequent study (Page et al., 2017) leaving the role of PTM in chloroplast signalling questionable.

PEX2 is a peroxisome membrane protein with a cytosolically exposed RING domain E3 ligase that regulates the recycling and turnover of the PEX5 import receptor through ubiquitination (Burkhart *et al.*, 2014). Interestingly a mutant of Arabidopsis *PEX2* (*ted3)* was recovered as a suppressor of the photomorphogenesis mutant *det1* (Hu *et al.*, 2002). The mechanism of this remains unknown but an artificially expressed RING domain was found in the nucleus where it interacted with the transcription factor HY5 (Desai *et al.*, 2014). Possible mechanisms could be cleavage of the RING domain and relocation to the nucleus, alternative transcription/translation sites or direct movement between peroxisome and nuclear membrane. Since peroxisomes are important nodes in the cell's antioxidant network and import is under redox control we speculate that PEX2 relocation could represent a potential mechanism for sensing the redox state of peroxisomes and relaying this information to the nucleus.

The above list is not exhaustive and it may in fact be the tip of the iceberg because there are many proteins in the literature that are suggested to undergo inter-compartmental switching in response to appropriate triggers. Arabidopsis hexokinase 1, for example, which is located at

the outer mitochondrial membrane, has been suggested to translocate between mitochondrion and nucleus, upon perception of sugar signals or methyl-jasmonate, in a manner that is linked to mitochondrial ROS production (Claeyssen and Rivoal, 2007; Xiang *et al.*, 2011).

Organelle movement and contact as a mechanism of protein movement

Apart from release of proteins from membranes, prevention of import into or promotion of export from organelles, direct transfer of proteins between membrane bound compartments via membrane extensions and contact sites can occur (Pérez-Sancho *et al.*, 2016) (**Figure 3**). The cytoplasm in plant cells is densely packed and mainly constrained by the vacuole and ER to a narrow cortical zone. Protein transfer between organelles requires regulated release and redirection. Redirection through the cytosol may be slow and prevent bulk delivery. Emerging evidence suggests that the physical interaction between organelles is a requirement for the exchange of small molecules, lipids and proteins in plants as well as in mammals and yeast (Cohen *et al.*, 2018). Coordinated re-arrangement of organelle positioning within the cell could provide a mechanism for shuttling moonlighting proteins between compartments. Targeted 'protected' delivery from degradation, or potential reversal of the PTM, could be provided through the formation of a micro-environment between organelles that allows for exchanging proteins through a narrow 10-40nm cytoplasmic zone at the membrane contact site interface. Repositioning of organelles could also allow neighbouring organelles to signal to one another to regulate protein exchange.

Redox-dependent formation of stromules, matrixules and peroxules

Chloroplasts, mitochondria and peroxisomes are pleomorphic, dynamic organelles that produce tubules upon stress. Like membrane contact sites (MCS) these tubules allow positioning of the organelles in relation to each other within the cell and might be involved in the exchange of metabolites or macromolecules. For example, stroma-filled tubules called stromules **(Figure 4)** extend from the envelope of all plastid types. ROS increase peroxisome speed, resulting in membrane extensions (peroxules), which could facilitate contact with other organelles including chloroplasts (Gao *et al.*, 2016; Rodriguez-Serrano *et al.*, 2016; Rodriguez-Serrano *et al.*, 2009). However, the cargo of the tubular structures and the nature of the potential signals (metabolic or proteinaceous) that are released is largely unknown (Hanson and Hines, 2018). Dynamin-type proteins are thought to be involve in stromule formation, as well as in the formation of vesicles that are shed from the stromule tips (Hanson and Hines, 2018). At

least some of the plastid-derived vesicles are found in the vacuole, where they fulfil a role in chloroplast degradation.

Stromules allow actin-mediated anchoring of chloroplasts at different locations within the cell to facilitate specific functions. For example, they can extend along microtubules to guide chloroplast movement to the nucleus during innate immunity responses. The application of hydrogen peroxide (H_2O_2) resulted in rapid stromule formation in Arabidopsis leaves (Caplan *et al.*, 2015). The accumulation of ROS, like other pro-defense molecules, is sufficient to induce stromule formation leading to the development of direct contact points between the chloroplasts and nuclei (Caplan *et al.*, 2015). In addition, other direct contact sites between chloroplasts and nuclei that are induced by high light have been suggested to allow movement of H_2O_2 to the nucleus from attached chloroplasts (Exposito-Rodriguez *et al.*, 2017). Arogenate dehydratase (ADT) 2 which catalyzes the final step in phenylalanine biosynthesis localizes to stromules and also helps in dividing chloroplasts, whilst ADT5 is proposed to traffic to nuclei via stromules, (Bross *et al.*, 2017). Another interesting example of possible organelle to organelle transport of proteins via membrane extensions is the triacyl glycerol lipase SDP1 which is proposed to move from peroxisomes to oil bodies in a tubule- and retromer- dependent process (Thazar-Poulot *et al.*, 2015).

Mitochondria produce structures that are partly homologous to the chloroplast stromules, in response to light and other stimuli in an endoplasmic reticulum (ER)-mediated manner (Schmidt *et al.*, 2016). The protrusion-driven movement and positioning is considered to promote the inter-compartmental trafficking of metabolites and proteins but there remains a paucity of data on which proteins are trafficked and the mechanisms involved. ROS and redox cues modify microtubule orientation and behaviour within cells, as well as the operation of protein import and export machineries (Schmidt *et al.*, 2016). So far, it remains to be determined if these organelle-derived tubular structures are involved in direct exchange of metabolites or macromolecules between compartments, or might rather have a supportive function in the communication between organelles by acting as an cellular anchor to temporary fix their relative position to each other.

Conclusions and Perspectives

Our understanding of ROS functions has been entirely revised in recent decades. Initially confined to oxidative stress and associated cellular damage ROS are now recognised as signals

released from the plasma membrane and organelles to orchestrate plant growth and stress tolerance. Moreover, the same oxidative changes to proteins such as irreversible oxidation, nitrosylation of glutahionylation of cysteine residues that were once regarded as damage are now recognised as being instrumental in regulating protein-protein interactions and signalling. Little is known about how protein carbonylation functions as a PTM in response the cellular redox changes. Literature evidence supports the concept that changes in ROS production alter the redox status of plant cells, exerting a strong influence on metabolism and gene expression. Redox-related posttranslational modifications may have important effects on chromatin structure and function, opening up a new area of redox epigenetics (García-Giménez *et al.*, 2012). Histone PTMs have a direct impact on chromatin conformation, controlling important cellular events such as cell proliferation and differentiation. The carbonylation of specific histones (H1, H10, and H3.1 dimers) has been described during DNA synthesis in proliferating in NIH3T3 fibroblasts was found to decrease when nuclear proteasome activity was activated. suggesting that this PTM prevent excessive histone accumulation during DNA synthesis (García-Giménez *et al.*, 2012).

Until recently the paucity of experimental data on subcellular protein distribution has limited our understanding of the capacity and ability of proteins to move between different intracellular compartments. There has been a step change in our knowledge of proteins that perform more than one cellular function. The term given to such proteins is 'moonlighting', but this description is limited as we have discussed above because it does not apply to all proteins that move between different cellular compartments. Moreover, it has become increasingly apparent that protein localisation is not fixed and a high proportion of cellular proteins have the potential to move between compartments in response to specific triggers. In some cases this movement is the basis for an alternative cellular function. At present however we have only a fragmented picture with relatively few well characterised examples of proteins in plants that change compartment in order to moonlight, and the mechanisms by which they do so are largely unexplored. Here we have presented evidence in support of an extension to existing concepts suggesting that redox PTMs are likely to be a key driver for inter-compartmental shifts of antioxidant and redox-regulated proteins. Redox cues and associated PTMs are fundamental regulators of alternative protein functions and localization. However, the extent of this phenomenon, what makes proteins move and the mechanisms by which they do so remains largely obscure. Redox-regulated PTMs that drive intercompartmental protein relocation have the potential to integrate metabolic processes and influence genetic and epigenetic controls of

plant growth and stress tolerance. This prospect is already opening a new intriguing and technically-challenging area of research.

Although it is widely recognised that ROS act as a signals through the redox processing of other molecules particularly proteins. Reactively little is known about the network of proteins that are undergo redox-mediated PTMs, highlighting the need for improved redox proteomics approaches. Moreover, a larger tool box of molecular and cell biology techniques is required to fully understand the redox-mediated movement of organelles and the associations/dissociations between different cellular compartments, as well as if and how redox-mediated structural changes facilitate direct movement of proteins from one compartment to another, particularly between chloroplasts, mitochondria, peroxisomes and nuclei without the need to transverse the cytosol in-between.

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FIGURE LEGENDS

Figure 1 Potential mechanisms of protein relocation

Proteins can potentially change their cellular localisation by a number of mechanisms. Proteins which have been inserted into an organelle membrane can be released by regulated proteolysis as described for the chloroplast envelope localised PTM1 and ER membrane localised ANAC013 and ANAC017. All organelles appear to have an ER associated degradation (ERAD)-like pathway which exports proteins in an ubiquitin dependent manner for degradation by the proteasome. Whether proteins can be exported and escape degradation to be retargeted elsewhere in the cell is currently unknown. Ubquitination on membrane components can also lead to organelle turnover. Transport by direct organelle contacts is also a possible mechanism. Proteins normally targeted to an organelle can be prevented from import through either modification of the import machinery or modification of the cyctosolic precursor form of the protein. This can include post translational modifications (PTMs) which can modify the targeting signal or affect interactions with other binding partners. See text for further details.

Figure 2 Switching partners: model for regulation of catalase localisation through interaction with different binding proteins.

Several cytosolic proteins have been reported to interact with plant catalases. Redox mediated PTMs could alter the affinity of catalase for different binding partners leading to a change in distribution between peroxisomes, cytosol and nucleus. See text for further details.

Figure 3 Organelle interactions through protrusions and membrane contact sites

Organelle-organelle interactions in cells occurs by either the formation of membrane contact sites (MCS) between organelles or by the formation of tubular structures by one organelle. MCSs are known to occur between mitochondria and plastids, mitochondria and the ER, and plastids and the ER. In addition, mitochondria, plastids and peroxisomes form tubular structures. In the case of plastids stromules are formed especially in the direction of the nucleus. Mitochondria form matrixules within ER structures and peroxisomes form peroxules in the vicinity of plastids, mitochondria and the nucleus. Both MCS and the tubular structures will mediate communications between organelles by exchanging signaling molecules, metabolites or potentially even proteins.

Figure 4. Stromule formation in *N. benthamiana* leaves upon transient over-expression of GFP-tagged plastid outer membrane protein AtLACS9 (At1g77560).

A. tumefaciens carrying AtLACS9-GFP (Breuers *et al.*, 2012) and a second strain carrying the P19 silencing suppressor construct (Takeda *et al.*, 2002) were co-infiltrated at 0.4 OD each into 7-week-old *N. benthamiana* leaves. Fluorescence imaging was done at 72 hrs. post infiltration with a Zeiss LSM710 confocal microscope. Image is a maximum projection of 10 optical sections. GFP (green); chlorophyll autofluorescence (red).

Figure 5. Methods to identify the sulfenome. A. Protein-based probe YAP1C. B. Small molecule-based probe DYn-2. See Box I text for detail.

Box I - key developments to help understand reversible oxidative modifications in plants.

1. Genetically-encoded protein-based tools to trap sulfenylated proteins *in situ*.

S-Sulfenylation (protein-SOH) is a reversible oxidative PTM that acts as regulatory switch in signal transduction pathways. However the global "sulfenome" is particularly challenging to detect as this PTM is transient, unstable, and prone to over-oxidation even during cell lysis. Recently a genetically-encoded tool to capture *S*-sulfenylated proteins was developed (Waszczak *et al.*, 2014). The cysteine-rich domain of the yeast transcription factor YAP1 forms disulfides with *S*-sulfenic acid modifications on its cognate signalling protein; fusion of this domain with an affinity tag creates a tool to capture and enrich *S*-sulfenylated proteins *in vivo* (Figure 5, **A**). YAP1 can be expressed in cells, with control cells expressing a catalytically inactive version (YAP1A), and, following cell lysis, downstream affinity purification used to identify disulfide linked proteins. The authors detected ~100 sulfenylated proteins in Arabidopsis cell suspensions exposed to H_2O_2 oxidative stress (Waszczak *et al.*, 2014).

2. Small molecule-based probes to detect the sulfenome.

A complementary approach exploits the chemoselective reaction of small molecules based on dimedone with sulfenic acid. Whilst YAP1C recognition of sulfenic acids is dependent on protein-protein interactions, a small molecule is in principle more general and able to access more sulfenylation sites. The Carroll group have pioneered the use of DYn-2, a dimedone probe that is small yet ready appended to affinity tags such as biotin by click chemistry for enrichment of sulfenylated proteins (Figure 5, **B**) (Paulsen *et al.*, 2011). Akter et al. applied DYn-2 is Arabidopsis cultures (Akter *et al.*, 2015), identifying 226 sulfenylation events in response to oxidative stress, and, more recently, in plants (Akter *et al.*, 2017).

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Table 1 Moonlighting proteins in plants

Protein	Function	Location	Moonlighting function	Reference
PUMPKIN	Plastid UMP kinase	plastid	RNA binding Plastid transcript introns	(Schmid *et al.*, 2019)
WHIRLY1	Nuclear encoded transcription factor involved in pathogen response	Nucleus/ plastid	RNA processing in the plastid	(Foyer *et al.*, 2014; Isemer *et al.*, 2012)
PEX2	Ubiquitin E3 ligase	Peroxisome membrane/ nucleus?	*ted3* gain of function mutant suppresses photomorphogenesis mutant *det1* and evidence for interaction with Hy5 TF in nucleus but mechanism/function unknown	(Desai *et al.*, 2014; Hu *et al.*, 2002)
Catalase	Antioxidant enzyme	Peroxisome matrix, cytosol	Hijacked to nucleus by plant pathogens to modulate cell death but mechanism unknown	(Zhang *et al.*, 2015)
MSH1	Required for organelle genome stability	Plastid and mitochondrial targeted	Alteration in nuclear DNA methylation	(Virdi *et al.*, 2015)
pdNAD-MDH	NAD-dependent malate dehydrogenase	Plastid	Activity independent stabilisation of FtsH12 component of inner envelope membrane protease AAA-ATPase complex. Essential for viability	(Schreier *et al.*, 2018)
AROGENATE DEHYDRATASE2/5	Phenyl alanine Biosynthesis	Stroma and stromules	Interaction with chloroplast division machinery. ADT5 isoform located in nucleus	(Bross *et al.*, 2017)
GAPDH isoforms	Glycolysis Calvin cycle	cytosol	Redox sensitive protein accumulating in the nucleus under stress conditions	(Zaffagnini *et al.*, 2013) (Yang and Zhai, 2017).
LSD1	Forms redox dependent interaction with a suite of proteins affecting cell division vs cell death	Cytosol nucleus	Transcriptional activator	(Czarnocka *et al.*, 2017)
ACO1, aconitase	Citrate metabolism, mRNA binding	Cytosol	TCA cycle enzyme and mRNA binding protein to promote translation of CSD2	(Moeder *et al.*, 2007)